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Doxorubicin-induced DNA intercalation and scavenging by nuclear glutathione *S*-transferase π

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ABSTRACT Glutathione *S*-transferase (GST) functions in xenobiotic biotransformation and drug metabolism. Increased expression of GSTπ, an isozyme of GST, has been found in cancer cells resistant to doxorubicin hydrochloride (DOX) or *cis*-diamminedichloroplatinum (II) (CDDP), and this increase was believed to be correlated with drug resistance of cancer cells. GST is mainly expressed in the cytoplasm; GSTπ in the nucleus has been reported in cancer cells, but the meaning of this result is not known. Here, we studied changes in the amount of nuclear GSTπ after exposure of cancer cells to anticancer drugs, and role of the nuclear GSTπ in drug resistance. We found nuclear GSTπ in cancer cells resistant to DOX, and the amount of nuclear GSTπ was enhanced by treatment of the cancer cells with DOX or CDDP. We also found that a mushroom lectin, an inhibitor of nuclear transport, inhibited the nuclear transfer of GSTπ, suggesting the existence of a specific transport system for the nuclear transfer of GSTπ. Nuclear GSTπ protected DNA against damage by anticancer drugs. These results suggest a possible role of GSTπ in the acquisition of resistance to anticancer drugs by cancer cells. —Goto, S., Ihara, Y., Urata, Y., Izumi, S., Abe, K., Koji, T., Kondo, T. Doxorubicin-induced DNA intercalation and scavenging by nuclear glutathione *S*-transferase π. *FASEB J.* 15, 2702–2714 (2001)

Key Words: Glutathione S-transferase π · doxorubicin · cisplatin · nuclear transfer · DNA damage

GLUTATHIONE *S*-TRANSFERASE (GST, EC 2.5.1.18) is mainly expressed in cytoplasm and is ubiquitous in nature. GST functions in xenobiotic biotransformation (1), drug metabolism (2), protection against peroxidative stress of lipids and nucleic acids (3–5), and isomeration of prostaglandins (6). Human GSTπ is one of a family of GSTs. Increases in the expression of GSTπ have been reported in various human cancer tissues and precancerous tissues, and GSTπ has been employed in cancer research as a tumor marker (7–11). An increase was also found in cancer cell lines resistant to doxorubicin hydrochloride (DOX), *cis*-diamminedichloroplatinum (II) (CDDP) (12–14), or alkylating agents (15). GSTπ in the nucleus has been reported in uterine cancer cells (16) and glioma cells (17), suggesting a negative correlation between the existence of GSTπ in the nucleus of cancer cells and patient survival. However, there has been no report on the mechanisms related to the presence of GSTπ in the nucleus or on the physiological role of nuclear GSTπ.

DOX is an anticancer drug that inhibits topoisomerase II. DOX interferes with the topoisomerase II-DNA complex, leading to the formation of double-stranded breaks of DNA or direct intercalation with DNA, which in turn inhibits DNA duplication and transcription for mRNA (18). Production of reactive oxygen species (ROS) is also thought to be a cytotoxic effect of DOX on cancer cells (19).

CDDP, a platinum-containing drug, binds to DNA guanine residues and forms cross-linkages inside or among the DNA chains (20). The cross-linkage of DNA by CDDP causes a change in the structure of the DNA and inhibits the transcriptional activity to form mRNA. The DNA coupled with CDDP is recognized by proteins with high mobility group domains, to which repair enzymes are unable to bind and repair injured DNA. These changes finally lead to cancer cell death (21, 22).

There have been many reports on the increase in the intracellular reduced form of glutathione (GSH) in cancer cells resistant to DOX and CDDP (23–26). GSH is synthesized by the activity of two ATP-requiring enzymes, γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase. Elevated levels of γ-GCS mRNA and its protein have been reported to correlate with acquisition of resistance to DOX and CDDP (25, 26). Treatment of cancer cells with buthionine sulfoximine (BSO), a specific inhibitor of γ-GCS, decreases the level of GSH and increases the sensitivity to anticancer drugs in vitro (23, 24). GSH is thought to associate with the efflux system of DOX and CDDP through ATP-binding cassette transporters (ABCs), such as canalicular multispecific organic anion transporter (cMOAT), and multidrug resistance-associated protein 1 (MRP1) (26–31). Depletion of intracellular GSH using BSO causes

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decrease in the efflux activity of DOX and CDDP, reducing the drug resistance of cancer cells (31, 32). We previously reported that GSTπ forms a CDDP-GSH adduct, which is transported outside the cells (33), and the efflux activity of CDDP is elevated in cancer cells resistant to CDDP (26). It has been reported that DOX forms an adduct with GSH inside the cells (34), although the mechanism for the formation of the DOX-GSH adduct is unknown.

Transfection of the GSTπ gene into cancer cells to overexpress the enzyme has resulted in an enhancement of resistance to DOX and CDDP (35–38); other reports have neglected the effect of transfection of GSTπ into cancer cells on drug resistance (39). These studies did not examine the nuclear localization of GSTπ. In our study here, we found that GSTπ was present in the cytoplasm of all cancer cell lines examined, but nuclear GSTπ was not detected in some cell lines. The amount of GSTπ in the nucleus seemed to correlate with drug resistance. Specifically, we addressed the following:

1) Change in the amount of nuclear GSTπ after exposure of cancer cells to anticancer drugs

2) Regulation and mechanism of the nuclear transfer of GSTπ

3) Role of nuclear GSTπ in drug resistance

MATERIALS AND METHODS

Materials

CDDP was a gift from Nihon Kayaku (Tokyo, Japan), and 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxy-camptothecin (CPT-11) was from Yakult Honsha (Tokyo, Japan). DOX and edible mushroom (*Agaricus bisporus*) lectin (ABL) were purchased from Wako Pure Chemical Industries (Osaka, Japan); RPMI 1640, Dulbecco's modified Eagle's medium (DMEM), and FBS were from Gibco BRL (Rockville, MD); horseradish peroxidase-labeled anti-rabbit IgG was from DAKO A/S (Glostrup, Denmark); anti-human p53 monoclonal antibody was from Oncogene Research Products (Cambridge, MA); fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG was from ICN Pharmaceuticals (Aurora, OH); Slow Fade Light Antifade kit was from Molecular Probes (Eugene, OR); and Enhanced Chemiluminescence (ECL) kit was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Other chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO). Anti-human Cu,Zn-superoxide dismutase (Cu,Zn-SOD) polyclonal antibody was a gift from Dr. K. Suzuki (Hyogo College of Medicine, Nishinomiya, Japan).

Preparation of cells

We used the human cancer cell lines HCT8 (colonic carcinoma), T98G (glioblastoma), PC-6 (small cell lung carcinoma), A549 (lung adenocarcinoma), A2780 (ovary carcinoma), and THP-1 (acute monocytic leukemia). Dr. K. J. Scanlon (Berlex Biosciences, CA) donated HCT8 and A2780 cells, and Dr. H. Isobe (Hokkaido University School of Medicine, Sapporo, Japan), the A549 and PC-6 cells. T98G and THP-1 cells were purchased from American Type Culture Collection (Rockville, MD). T98G cells were maintained in DMEM, and the other cells were maintained in RPMI 1640. They were supplemented with 10% FBS at 37°C in 5% CO_2 with 100% humidity. Six hours before treatment with anticancer drugs or ABL, the medium of cells with 10% FBS was changed to medium with 1% FBS. About 2×10^6 cells were harvested with trypsin and washed twice with PBS (0.137 M NaCl, 2.68 mM KCl, and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, PBS) at 4°C. The pellets were stored at −80°C before use. The doses of anticancer drugs used in this study were based on the results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the trypan blue dye exclusion test. HCT8 cells were treated with various concentrations of anticancer drugs for 24 h. The doses that suppressed cell growth by 30–50% and that killed less than 6% of cells were determined.

Preparation of cytoplasmic and nuclear proteins

The cytoplasmic and nuclear proteins were prepared as described by Dignam et al. (40). Briefly, cell pellets (1×10^6 cells) were treated with 100 μl of hypotonic buffer (10 mM HEPES at pH 7.8, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride [PMSF], 2 μg/ml pepstatin, 2 μg/ml leupeptin). After centrifugation of the sample (1800*g*, 4°C, 1 min), the supernatant was prepared as the cytoplasmic fraction. The debris was washed three times with the hypotonic buffer, treated with 100 μl of 50 mM HEPES (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM PMSF, 2 μg/ml pepstatin, and 2 μg/ml leupeptin, and then gently rotated with a rotator at 4°C for 30 min. The supernatant was prepared as the nuclear fraction.

Preparation of GSTπ antibody

GSTπ was purified from human placenta, and polyclonal antibody against human GSTπ was obtained by immunization of rabbits with the purified GSTπ as described previously (33).

Immunological assay

Immunological levels of GSTπ, p53, and Cu,Zn-SOD in the cells were estimated by Western blotting. Lysate from the extract of 1×10^5 cells was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel, transferred to a nitrocellulose membrane, and immunologically stained with rabbit anti-human GSTπ IgG, mouse anti-human p53 IgG, or rabbit anti-human Cu,Zn-SOD IgG as the first antibody, and then with horseradish peroxidase-labeled anti-rabbit IgG as the second antibody. Blots were developed by enhanced chemiluminescence using the ECL kit, and relative immunological activities were analyzed by NIH Imaging software. The protein concentration was determined according to Redinbaugh and Turley (41), with bovine serum albumin as the standard.

Phase-contrast microscopy

The effect of anticancer drugs on the morphological change in cell shape was estimated by phase-contrast microscopy (Nikon TMD300, Tokyo, Japan).

Immunohistochemistry

For immunostaining, HCT8 cells were maintained with RPMI 1640 containing 10% FBS in a four-well Lab Tec Chamber

were washed three times with PBS and were fixed with 3% paraformaldehyde/PBS for 20 min. Chambers were rewashed three times with PBS and then treated with 1% Triton-X 100/PBS for 10 min. After cells were washed again three times with PBS and blocked with 3% bovine serum albumin/PBS for 1 h at room temperature, they were treated with anti-GSTπ for 1h. After cells were washed with PBS, they were treated with FITC-conjugated anti-rabbit IgG for 1 h, avoiding exposure to light, and then were washed with PBS and treated with 50 μg/ml propidium iodide/PBS for 10 min. They were washed with PBS and mounted in glycerol/PBS conta antifade reagent. Fluorescence intensity was observed an Axioskop2 fluorescence microscope (Carl Zeiss, Germany), and the findings were analyzed by using a camera (AxioCam) and AxioVison software.

TUNEL assay and cell cycle analysis

Terminal deoxynucleotidyl transferase (TdT)-mediated dU biotin nick end-labeling (TUNEL) assay was performed by u an Apop Tag Plus Fluorescein In Situ Apoptosis Detection

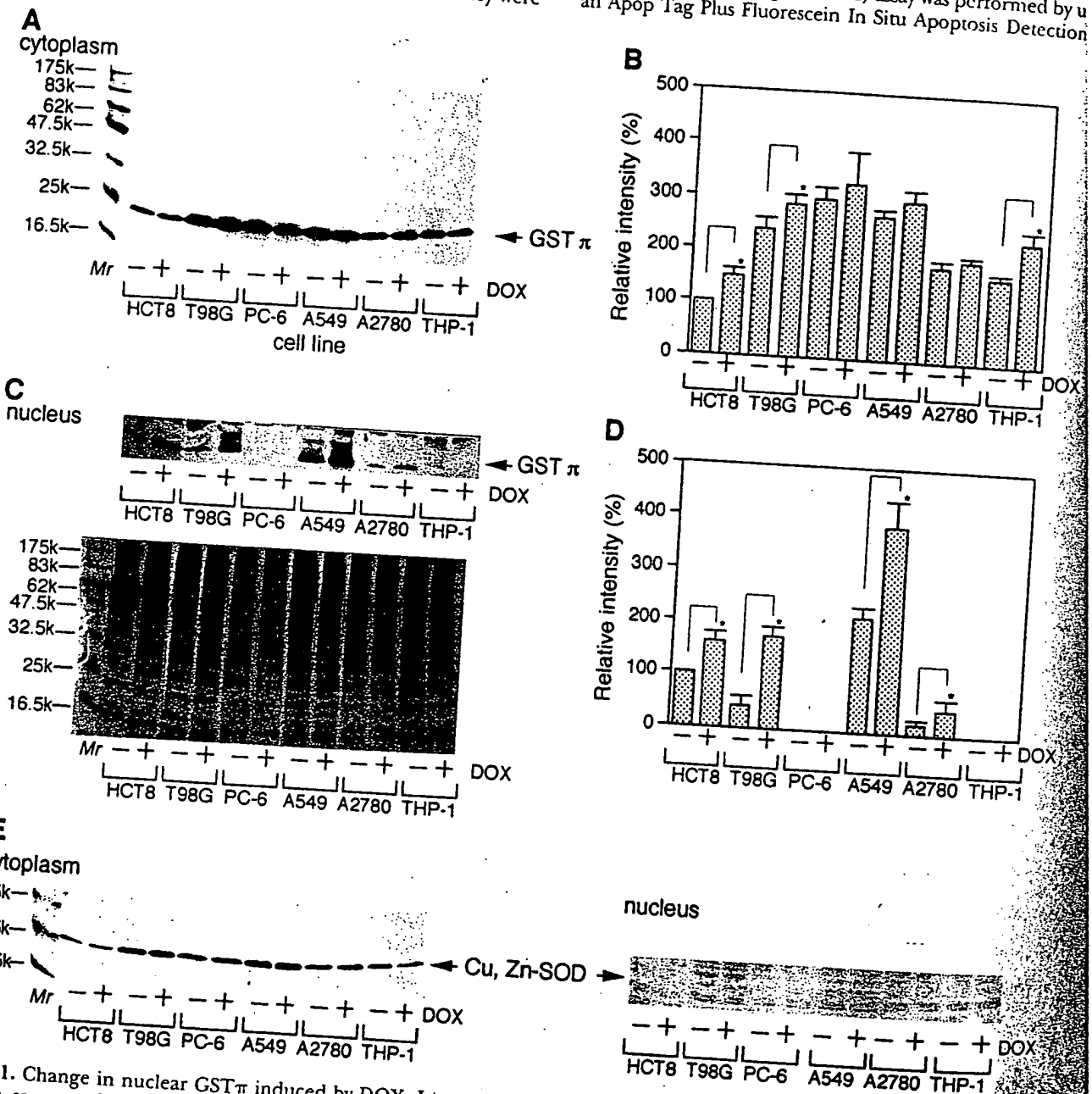


Figure 1. Change in nuclear GSTπ induced by DOX. Immunological activity of GSTπ was estimated by Western blot analysis. Human cancer cells were incubated for 6 h in the presence (+) or absence (−) of 10 μM DOX. Proteins prepared were separated by SDS-PAGE in a 12.5% gel, transferred to a nitrocellulose membrane, and immunologically stained with anti-GSTπ rabbit IgG. Molecular weight standards (M_r, lane 1) are as noted. *A*) Cytoplasmic GSTπ. *B*) Relative intensity in each lane corresponds to the cell lines as described in *A*. Values are expressed as relative to the intensity in HCT8 cells without DOX as 100%. *C*) Nuclear GSTπ. Nuclear proteins from 1×10^5 cells were applied to a SDS-PAGE gel. The upper panel shows Western blots of GSTπ. For the lower panel, under the same conditions as for the immunoblots, the gel was stained using Coomassie brilliant blue. *D*) Each lane corresponds to that in the upper panel of *C*. Data are the means of three independent analyses; bars show the SD. $^*P < 0.05$ compared with each cell without DOX treatment. *E*) Immunological activity of Cu,Zn-SOD was estimated in cytoplasm (left panel) and the nucleus (right panel).

ntergen, Purchase, NY). Briefly, about 2×10^6 cells were arvested, fixed in 70% ethanol, treated with TdT for 1 h and ıen FITC-conjugate antidigoxigenin for 1 h at room tempera- ıre, washed with 0.1% Triton X-100/PBS, and resuspended in ropidium iodide containing RNase A. Fluorescence intensity as estimated simultaneously, at FL-1 (530 nm) for the TUNEL ssay and at FL-2 (585 nm) for the cell cycle analysis using a ACScan flow cytometer (Becton Dickinson, San Jose, CA).

nzyme activity

he activity of caspase-3 was estimated by using a CPP32/ aspase-3 Colorimetric Protease Assay Kit (Medical & Biolog- al Laboratories, Nagoya, Japan). Briefly, according to the nanufacturer's instructions, cell pellets were treated with ce-cold cell lysis buffer for 10 min. After centrifugation, 100 ıg of the supernatant protein was treated with DEVD-*p*- iitronilide at 37°C for 2 h, and the change in absorbance at 00 nm was measured. The relative activity is expressed with he absorbance of control for each time point as 1. The ıctivity of GST was estimated as described using 1-chloro-2,4- linitrobenzene as substrate (42). One unit of GST activity is xpressed as 1 μmol substrate changed/min.

Concentration of DOX

The intracellular concentration of DOX was estimated fluoro- metrically. Cells treated with various concentrations of DOX for 1 h were harvested and washed twice with ice-cold PBS. The fluorescence intensity of the cells was estimated at FL-3 (630 nm) using a flow cytometer. The intranuclear concen- tration of DOX was determined by using cell pellets treated with 0.1% Triton X-100/PBS as assay material.

Plasmid construct and DNA damage assay

The γ-GCS heavy subunit probe (267 base pairs corresponding to nucleotides 54–320 of the rat kidney γ -GCS heavy subunit) was prepared by polymerase chain reaction using rat liver mRNA as template. This probe was used to prepare a full-length human γ-GCS heavy subunit cDNA by cloning a human foreskin cDNA library in λgt 11 (43). After the blunting of 764 base pairs of γ-GCS corresponding to nucleotides 865-1628 of human γ-GCS from the full-length γ-GCS cDNA, an 8-mer *Bam*HI linker was ligated. A pUC 19 vector digested by *Bam*HI was ligated to γ-GCS cDNA to construct the plasmid pUC-γ-GCS. DNA intercalation by DOX was assayed by using pUC-γ-GCS. Various concentra- tions of DOX (0–20 μM) were incubated in the presence or absence of GSTπ (0–3 U) and a GSH mixture (2 mM GSH, 2 U of glutathione reductase, and 2 mM NADPH) for 30 min at 37°C, followed by incubation with 400 ng of the pUC-γ-GCS plasmid for 30 min. Similarly, DNA cross-linking by CDDP was assayed by using pUC-γ-GCS. Various concentrations of CDDP (0–100 μM) were incubated in the presence or absence of GSTπ (0–3 U) and a GSH mixture for 1 h at 37°C, followed by incubation with 400 ng of the pUC-γ-GCS plasmid for 3, 6, or 12 h. Then, in each experiment, 200 ng of the pUC-γ-GCS was applied to a 1% agarose gel for electrophoresis at 15 mA for 40 min. Final detection was with ethidium bromide (0.5 μg/ml).

Cell viability

Cell number and viability were determined by the trypan blue dye exclusion method and MTT assay. Cells in PBS were treated with an equal vol of 0.4% trypan blue and allowed to stand at room temperature for 5 min. Chambers of the hemocytometer were then filled and the dead (blue-stained) cells were enumerated under a phase-contrast microscope. The MTT assay was performed as previously described (26).

Statistical analysis

Data were presented as means ± SD. Differences were examined by using Student's t test. A value of $P < 0.05$ was considered significant.

RESULTS

Nuclear transfer of GSTπ by DOX

Figure 1 shows Western blots for GSTπ in human cancer cell lines. Immunological activity of GSTπ was observed in the cytoplasm of every cancer cell tested. The immunological activity of GSTπ was relatively high in T98G, PC-6, and A549 cells compared with HCT8, A2780, and THP-1 cells (Fig. 1*A, B*). Immunological activity of GSTπ was observed in the nucleus of HCT8, T98G, A549, and A2780 cells, whereas it was not detected in PC-6 or THP-1 cells (Fig. 1*C*, upper panel). Treatment with DOX (10 μM) for 6 h resulted in an increase in the level of GSTπ in cytoplasm in every cell line (Fig. 1*A, B*). The treatment caused a nuclear transfer of GSTπ in HCT8, T98G, A549, and A2780 cells (Fig. 1*C, D*). The lower panel of Fig. 1*C* shows the

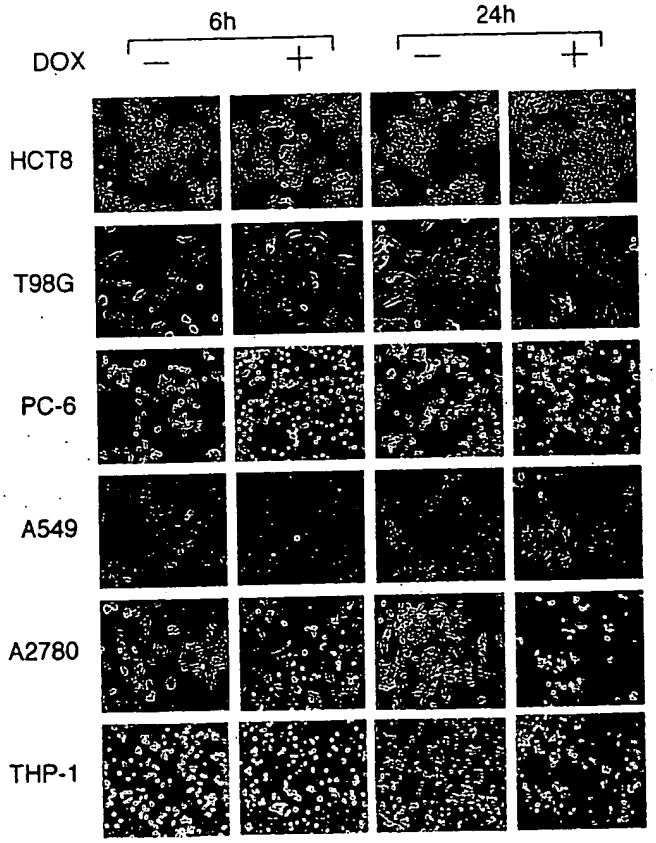


Figure 2. Change in cell shape. The morphological change in cell shape in the presence (+) or absence (−) of 10 μM DOX for 6 and 24 h was examined with a phase-contrast microscope (200 ×).

results of SDS-PAGE for total nuclear protein, suggesting that not all nuclear proteins were induced by treatment with DOX. To test for possible contamination by cytoplasmic protein, the amount of Cu,Zn-SOD was estimated by Western blotting. Apparent bands of Cu,Zn-SOD were detected in cytoplasmic fractions but not in nuclear fractions (Fig. 1*E*), ruling out the possibility of contamination by cytoplasmic proteins.

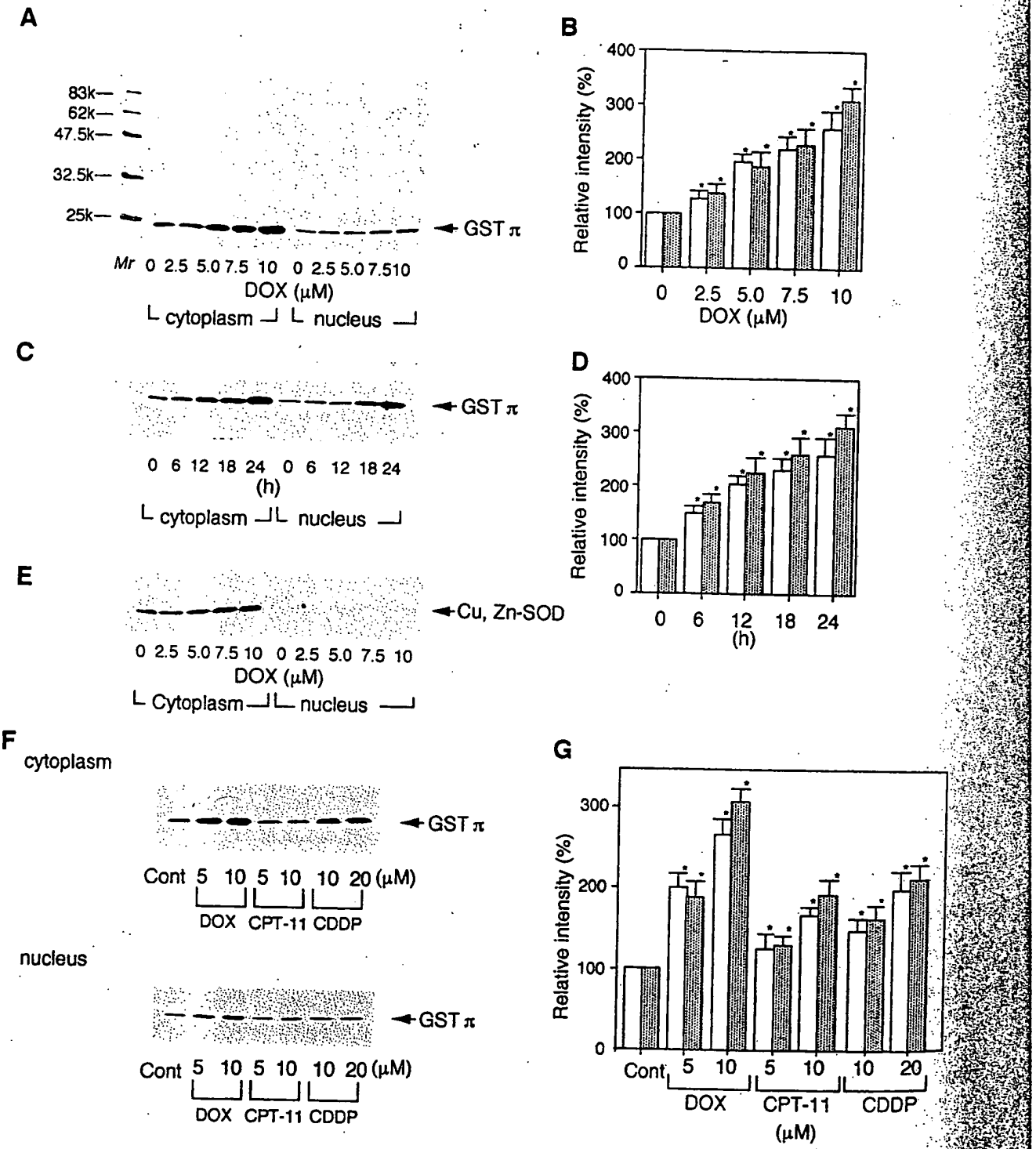


Figure 3. Change in immunological activity of GSTπ induced by DOX. HCT8 cells were incubated with various concentrations of DOX for 24 h, and the amount of GSTπ (*A*) and Cu,Zn-SOD (*E*) in cytoplasm and the nucleus was estimated by Western blot analysis. *B*) Relative intensity in each lane corresponds to lanes in *A*. Values are expressed as relative to the intensity in HCT8 cells without DOX as 100%. Open bars indicate GSTπ in the cytoplasm; dotted bars indicate GSTπ in the nucleus (*B, D, G*). *C*) The immunological activity of GSTπ in HCT8 cells incubated with 10 μM DOX for 0–24 h. *D*) Each lane corresponds to that in *C*. Values are expressed as relative to the intensity in HCT8 cells at 0 h as 100%. *F*) Change in the immunological activity of GSTπ induced by CPT-11 and CDDP. *G*) Each lane corresponds to that in *F*. Values are expressed as relative to the intensity in HCT8 cells without anticancer drugs as 100%. Data are means of three independent analyses; bars show the SD. $^*P < 0.05$ compared with control cells.

Morphological change by DOX

Figure 2 shows the morphological change in cell shape after treatment with 10 μM DOX for 6 and 24 h. HCT8, T98G, and A549 cells, in which GSTπ accumulated in the nucleus, revealed no apparent change in morphological features at 6 h, and only growth restriction at 24 h. The cells that adhered to the culture dish wall were somewhat hypertrophic at 24 h; however, not many round cells detached from the wall. In contrast, PC-6, A2780, and THP-1 cells, in which GSTπ did not accumulate in the nucleus, revealed a change in cell shape at 6 h; furthermore, most of the cells were rounded up and detached from the dish wall at 24 h. Cell viability analysis showed that the round cells detached from the dish wall were dead. Subsequent experiments were done with HCT8 cells.

Time and dose dependence of the change in GSTπ

Figure 3 shows results of Western blotting for GSTπ. DOX induced cytoplasmic GSTπ in a dose-dependent (Fig. 3*A, B*) and time-dependent (Fig. 3*C, D*) manner. A concomitant increase in the level of GSTπ was observed in the nucleus. There was no apparent difference in the electrophoretic pattern of GSTπ on the gel between cytoplasm and nucleus, which excludes the possibilities that the bands in the nucleus are of other proteins cross-reactive to anti-GSTπ antibody, or that GSTπ underwent alternative splicing or post-translational modification. During the experiment, no Cu,Zn-SOD was transferred to the nucleus (Fig. 3*E*).

Next, the nuclear transfer of GSTπ was examined with other anticancer drugs. CPT-11 and CDDP had an effect on the nuclear transfer of GSTπ corresponding to increase in GSTπ in cytoplasm (Fig. 3*F, G*).

Immunohistochemical staining of GSTπ

The intracellular localization of GSTπ was evaluated by immunohistochemistry (**Fig. 4**). An increase in the signal intensity for GSTπ was observed in the nucleus of HCT8 cells treated with DOX (10 μM), CPT-11 (10 μM), or CDDP (20 μM) for 6 h, with greater accumulation of the signal within 24 h. The data are consistent with those obtained by Western blotting (Figs. 2 and 3).

Effect of ABL

ABL, a lectin, is known to be internalized in intact cells and interfere with the transfer of nuclear proteins (44). The effect of ABL on the nuclear transfer of GSTπ was studied (**Fig. 5**). HCT8 cells preincubated with 40 μg/ml ABL for 10 h were treated with DOX for 6 h. Western blot analysis showed that pretreatment of the cells with ABL alone did not affect GSTπ in cytoplasm (Fig. 5*A*, upper panel, lanes 4 and 1). Pretreatment with ABL had no effect on the DOX (10 μM)-dependent induction of GSTπ in cytoplasm (Fig. 5*A*, upper panel, lanes 5 and 6 vs. 2 and 3). ABL alone decreased

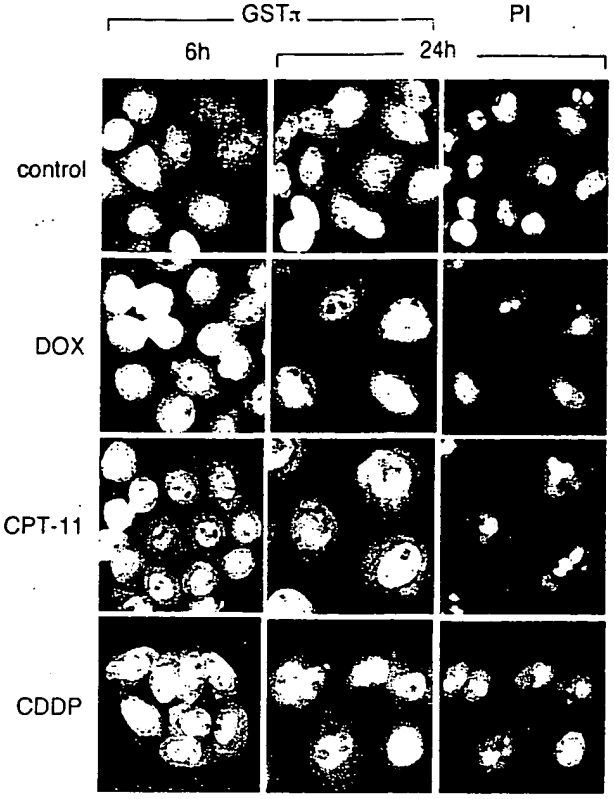


Figure 4. Intracellular localization of GSTπ. The presence of GSTπ was confirmed by immunological staining. HCT8 cells incubated with anticancer drugs for 6 or 24 h were treated with anti-GSTπ followed by treatment with FITC-conjugated anti-rabbit IgG for 1 h and propidium iodide (PI)/PBS for 10 min to clarify the nuclear region. The morphological characteristics of fluorescence intensity were observed with a fluorescence microscope (400×), and data were analyzed by using a CCD camera and AxioVison software. PI panels are shown for comparison.

the amount of GSTπ in the nucleus by 40% (Fig. 5*A*, lower panel, lanes 4 and 1). Pretreatment with ABL caused a loss in the amount of GSTπ in the nucleus by 50% of that in cells stimulated by 5 and 10 μM DOX (Fig. 5*A*, lower panel, lanes 5 and 6). Next, we examined the effects of hydrogen peroxide on the nuclear transfer of GSTπ. Treatment of HCT8 cells with both 100 and 200 μM hydrogen peroxide for 30 min caused nuclear translocation of GSTπ, and the amount of GSTπ in the nucleus increased for up to 90 min. ABL inhibited the transfer of GSTπ by hydrogen peroxide (Fig. 5*C, D*). The protein p53 is known to localize both in the nucleus and cytoplasm and to possess a nuclear localization signal (NLS). No inhibitory effect of ABL was observed on the nuclear transfer of p53 (Fig. 5*E*). Immunohistochemical analysis of GSTπ showed similar findings to those obtained by Western blotting (Fig. 5*F*).

Table 1 shows changes in the activity of GST in the cytoplasm and nucleus after treatment of HCT8 cells with DOX, CPT-11, and CDDP. Treatment with 10 μM DOX increased GST activity by 1.2-fold (6 h) and 2.4-fold (24 h) in the cytoplasm, and by 1.4-fold (6 h) and 2.2-fold (24 h) in the nucleus. CDDP (20 μM) also

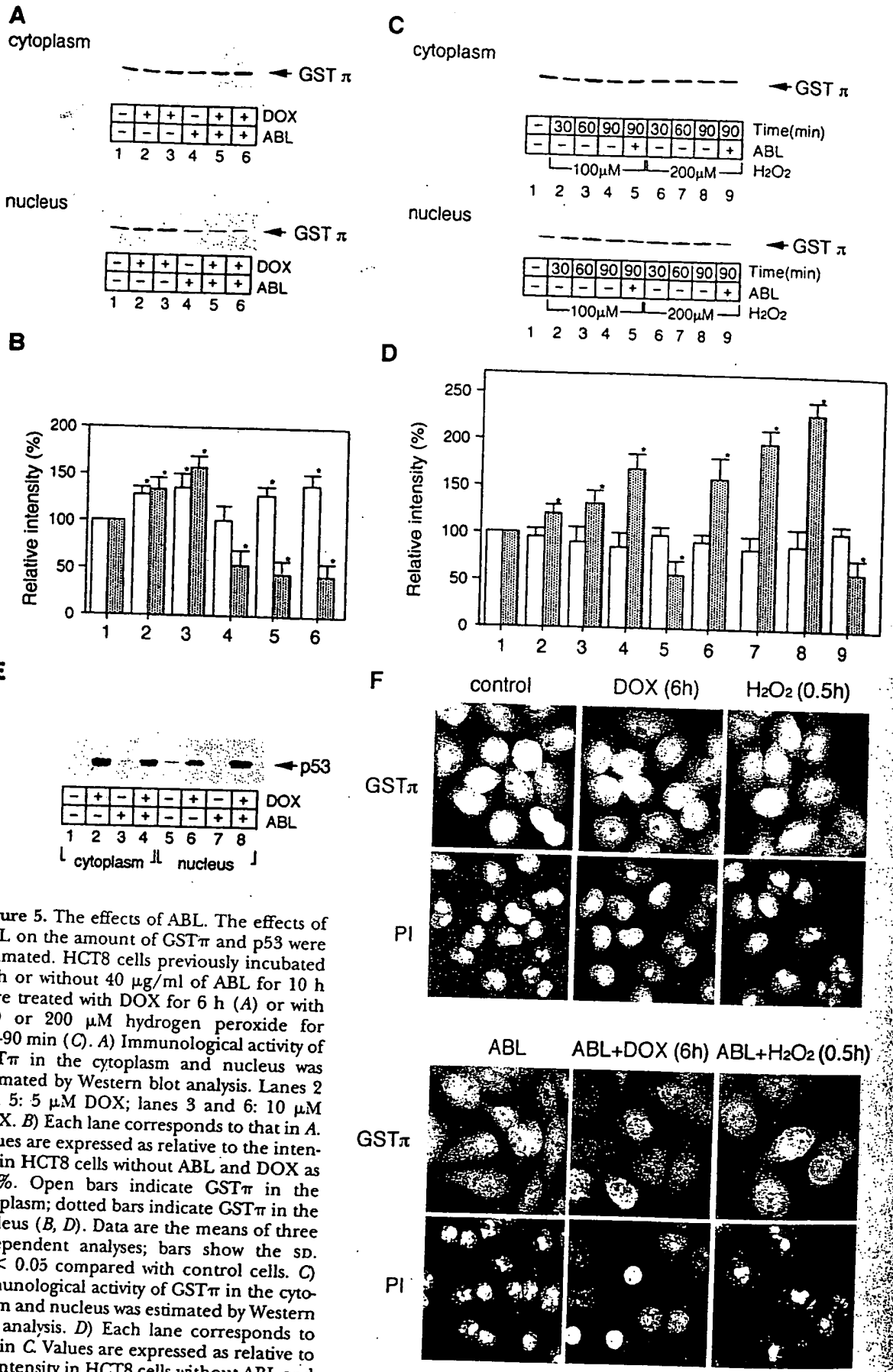


Figure 5. The effects of ABL. The effects of ABL on the amount of GSTπ and p53 were estimated. HCT8 cells previously incubated with or without 40 μg/ml of ABL for 10 h were treated with DOX for 6 h (*A*) or with 100 or 200 μM hydrogen peroxide for 30–90 min (*C*). *A*) Immunological activity of GSTπ in the cytoplasm and nucleus was estimated by Western blot analysis. Lanes 2 and 5: 5 μM DOX; lanes 3 and 6: 10 μM DOX. *B*) Each lane corresponds to that in *A*. Values are expressed as relative to the intensity in HCT8 cells without ABL and DOX as 100%. Open bars indicate GSTπ in the cytoplasm; dotted bars indicate GSTπ in the nucleus (*B, D*). Data are the means of three independent analyses; bars show the SD. $^*P < 0.05$ compared with control cells. *C*) Immunological activity of GSTπ in the cytoplasm and nucleus was estimated by Western blot analysis. *D*) Each lane corresponds to that in *C*. Values are expressed as relative to the intensity in HCT8 cells without ABL and hydrogen peroxide as 100%. Data are as in *B*. *E*) Immunostaining of p53 in the cytoplasm and nucleus. *F*) Effects of ABL on the intracellular localization of GSTπ were estimated by immunostaining as described in Fig. 4.

TABLE 1. *Change in the activity of GST in the cytoplasm and the nucleus*[a]

Treatment	GST (mU/10^6 cells)	
	Cytoplasm	Nucleus
Control	22.8 ± 1.6	5.2 ± 0.3
10 μM DOX (6 h)	28.6 ± 1.9	7.0 ± 0.4
10 μM DOX (24 h)	54.8 ± 2.5	11.5 ± 0.6
10 μM CPT-11 (6 h)	27.8 ± 1.5	7.2 ± 0.2
10 μM CPT-11 (24 h)	51.8 ± 2.8	9.5 ± 0.5
20 μM CDDP (6 h)	25.8 ± 1.2	6.7 ± 0.2
20 μM CDDP (24 h)	36.8 ± 2.1	9.0 ± 0.4
40 μg/ml ABL	21.5 ± 1.3	2.5 ± 0.3
40 μg/ml ABL + 10 μM DOX (6 h)	27.5 ± 1.2	2.0 ± 0.4

[a] Values are means ± SD of three independent experiments.

increased GST activity in the cytoplasm by 1.6-fold and in the nucleus by 1.7-fold at 24 h. Similarly, CPT-11 (10 μM) increased in GST activity in the cytoplasm by 2.3-fold and in the nucleus by 1.8-fold at 24 h. Pretreatment of cells with ABL abolished the DOX-stimulated GST activity in the nucleus at 6 h.

Effect of nuclear GSTπ on the cytotoxicity of DOX

To elucidate the physiological meaning of the nuclear GSTπ for cell morphology, TUNEL assay and cell cycle analyses were performed (**Fig. 6**). Morphologically, ABL treatment for 24 h increased the number of spindle-shaped cells. Pretreatment with ABL enhanced the DOX-induced change in shape at 6 h, the cells being round and detached from the culture dish wall at 24 h (Fig. 6*A*). With the TUNEL assay (Fig. 6*B*), ABL alone did not have any effect compared with the control cells. Pretreatment with ABL enhanced the DOX-induced increase in TUNEL-positive cells at 6 h, with an additional increase at 24 h. There was no difference in the cell cycle between ABL-treated cells and control cells. DOX (10 μM) treatment caused a G_2/M arrest at 6 h, and the number of arrested cells increased at 24 h. The cell cycle of the cells pretreated with ABL and then treated with DOX for 24 h was not measurable (Fig. 6*B*), possibly because of the enhancement of DNA fragmentation and a leak of DNA from the nucleus.

The effect of DOX on the caspase-3 activity was also evaluated (Fig. 6*C*). A threefold increase in the activity relative to the control was observed when cells were treated with 10 μM DOX for 24 h (lanes numbered 3). Pretreatment with ABL enhanced the DOX-induced activation of caspase-3 by 20-fold at 6 h and by 48-fold at 12 h, but the activity declined at 24 h (lanes numbered 4). Chromatin condensation in the nucleus examined with Hoechst 33342 was detected only in the nucleus treated with ABL and DOX (data not shown).

Accumulation of DOX in the nucleus

Accumulation of DOX was estimated by flow cytometry. **Figure 7** shows the effect of DOX in whole cells and the nucleus. Treatment with DOX increased the amount of DOX in whole cells in a dose-dependent manner (left panels). Pretreatment with ABL had no effect on the amount of DOX in whole cells. In the nucleus, the amount of DOX also depended on the dose (right panels). The amount was 240% at 50 μM DOX and 340% at 100 μM DOX, when that at 10 μM DOX was 100%. Pretreatment with ABL enhanced the amount of DOX in the nucleus by 13.6-fold of that obtained with 10 μM DOX alone.

Effect of GSTπ on DNA intercalation by DOX

The effects of GSTπ in the nucleus on DNA intercalation by DOX and DNA cross-linking by CDDP were studied in vitro by using a pUC-γ-GCS plasmid. **Figure 8** shows electrophoresis results with agarose gels for DOX (*A*) and CDDP (*B*). The electrophoretic pattern of pUC-γ-GCS plasmid indicated a relaxed form and a supercoiled form (Fig. 8*A*, lane 1). Treatment of the plasmid with DOX caused a relative decrease in the band corresponding to the supercoiled form. This decrease was apparent at 30 min, especially with 10 and 20 μM DOX (Fig. 8*A*, lanes 4 and 5). This change may due to a change in mobility of DNA in the gel and a decrease in the degree of DNA staining by ethidium bromide. In the presence of GSTπ and the GSH mixture, a broad band was observed between bands of the relaxed and supercoiled forms, and the intensity of the DNA bands increased (Fig. 8*A*, lanes 7–9). Removal of GSTπ or the GSH mixture resulted in a disappearance of the broad band, so that a similar pattern to that observed with DOX alone was found (Fig. 8*A*, lanes 10 and 11). Similarly, the effects of GSTπ on DNA cross-linking by CDDP were detected as changes in the electrophoretic patterns of two DNA bands, corresponding to the relaxed form by 20–100 μM CDDP for 6 h (Fig. 8*B*, lanes 6–8) and 12 h (Fig. 8*B*, lanes 10–12). In the presence of GSTπ and the GSH mixture, changes in the relaxed forms were restored to the control (Fig. 8*B*, lanes 14–16).

DISCUSSION

In the present study, we found evidence for the first time that

1) GSTπ existed in the nucleus in some cancer cells, and the amount of the nuclear GSTπ was enhanced by treatment of the cancer cells with anticancer drugs.

2) Among cancer cells in which the nuclear GSTπ was not observed, anticancer drugs did not induce expression of the nuclear GSTπ.

3) A specific transport system may exist for the nuclear transfer of GSTπ.

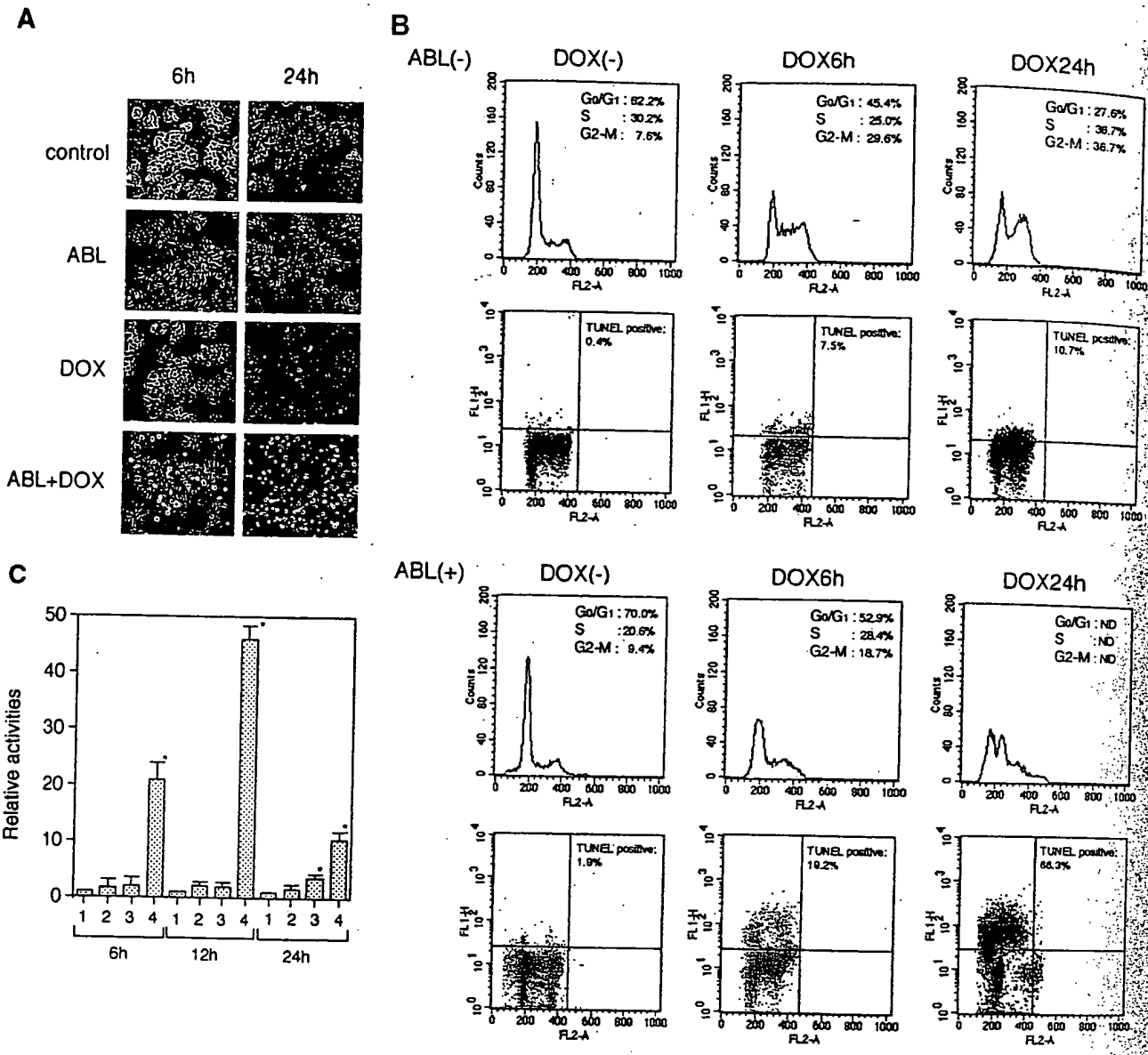


Figure 6. Cytotoxicity of DOX. The effect of ABL on the cytotoxicity of DOX was examined. *A*) The morphological change in cell shape after treatment of HCT8 cells with 40 μg/ml ABL for 10 h followed by 10 μM DOX for 6 or 24 h was examined by using a phase-contrast microscope (200 ×). *B*) The TUNEL assay was performed by using an Apop Tag Plus Fluorescein In Situ Apoptosis Detection Kit as described in Materials and Methods. HCT8 cells (2×10^6) treated as in *A* were employed for this assay. Control cells were incubated without ABL for 10 h and further incubated as indicated. Fluorescence intensity was estimated simultaneously for the TUNEL assay at FL-1 (530 nm) and for the cell cycle analysis at FL-2 (585 nm), using a FACScan flow cytometer. *C*) The effect of DOX on caspase-3 activity was estimated as described in Materials and Methods. Lanes numbered 1, control; 2, 40 μg/ml ABL alone for the indicated time period; 3, 10 μM DOX alone for the indicated time period; 4, 40 μg/ml ABL for 10 h followed by treatment with 10 μM DOX for the indicated time period. The relative activity was expressed with the absorbance of control at the indicated time as 1. Data are the means of three independent analyses; bars show the SD. $*P < 0.05$ compared with control cells.

4) GSTπ in the nucleus protected DNA from damage by anticancer drugs.

Pharmacological studies have suggested that GSTπ contributes to anticancer drug metabolism that attenuates cellular sensitivity to a drug (33, 45, 46). DOX is transported outside the cells through P-glycoprotein, MRP1, and other ABCs. Overexpression of these transporters causes a decrease in the intracellular amount of DOX, leading to resistance to DOX in cancer cells (29, 31, 32). Previous treatment of cancer cells with BSO, a specific inhibitor of γ-GCS, caused a decrease in the efflux of DOX (31). Transport of leukotriene C_4 through MRP1 was not inhibited by DOX alone, but the DOX-GSH adduct inhibited the transport of leukotriene C_4 in inside-out vesicles (29). These results suggest that ABCs recognize DOX in the form of a DOX-GSH adduct and that this adduct is necessary for efflux. The presence of the DOX-GSH adduct inside of cells was reported by Serafino et al. (34), suggesting that GSTπ plays a role in the formation of the adduct. However, there is no direct evidence of this. A possible role for GSTπ in the formation of the DOX-GSH adduct was

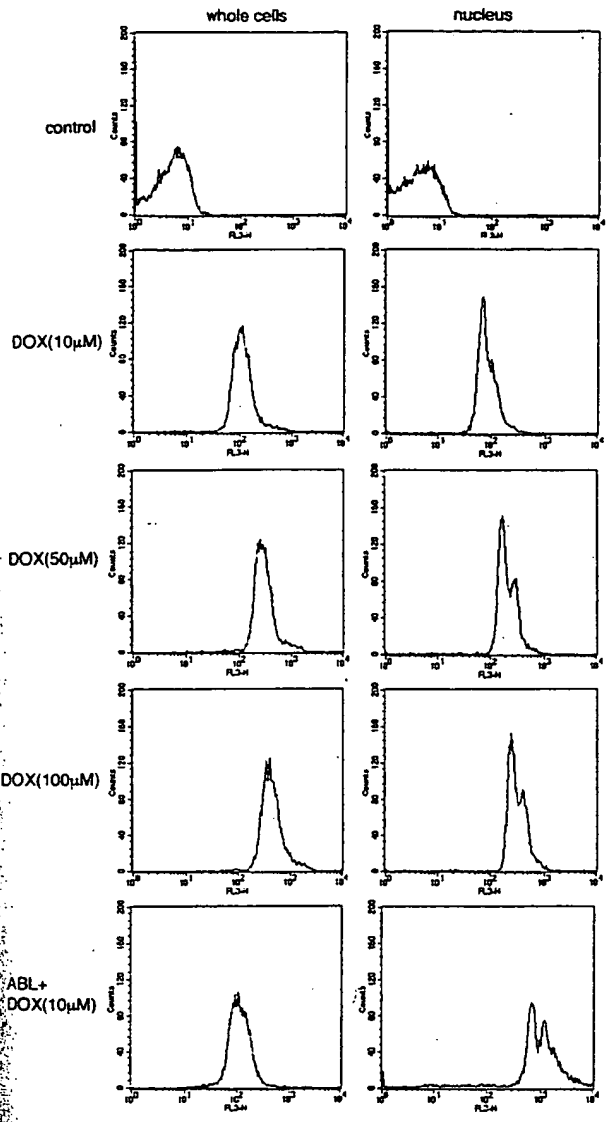


Figure 7. Accumulation of DOX in the nucleus. The accumulation of DOX in the whole cells and the intranuclear concentration of DOX were estimated by flow cytometry. Cells treated with various concentrations of DOX for 1 h were washed twice with ice-cold PBS. The fluorescence intensity of the cells was estimated at FL-3 (630 nm) using a flow cytometer. The intranuclear concentration of DOX was determined by using cell pellets treated with 0.1% Triton X-100/PBS as assay material. Left panels show the amount of DOX in whole cells, and right panels show that in the nucleus. The data are from typical analyses.

reported by Awasthi et al. (45) and Maeda et al. (46). Awasthi et al. treated cancer cells with ethacrynic acid, a specific substrate of GSTπ, which resulted in an inhibition of the formation of the DOX-GSH adduct and an enhanced cytotoxic effect of DOX (45). Maeda et al. treated cancer cells with W-77, an inhibitor of GSTπ, and found that it partially overcame the resistance to DOX (46).

CDDP is removed via ABCs such as MRP1 or cMOAT. Adduct formation of CDDP with GSH is necessary for the efflux of CDDP (26–28) catalyzed by GSTπ (33). These findings indicate that GSTπ is important in the first step of detoxifying CDDP in cancer cells. It has been reported that CPT-11 is transported via ABCs (47), but the role of GSTπ in CPT-11 metabolism is not known.

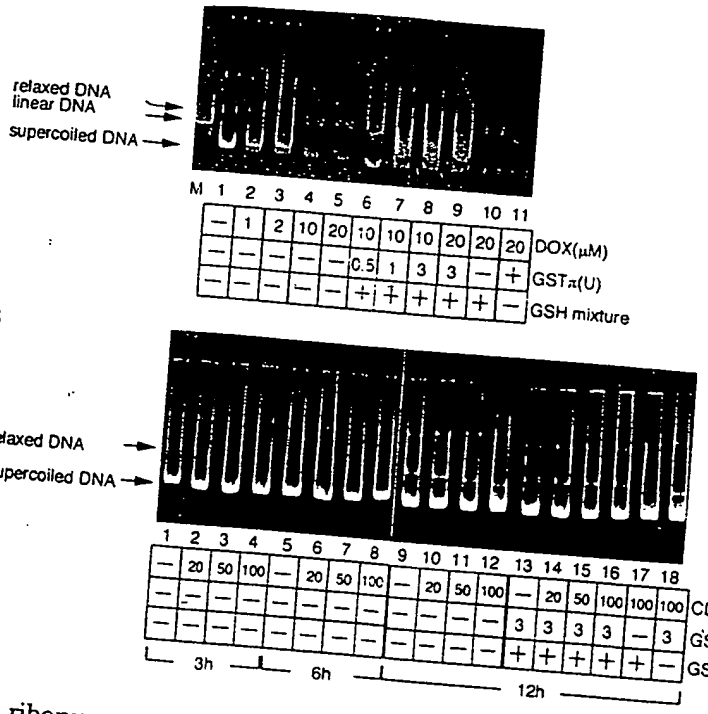
Transfection of other GST isozymes, such as GSTα or GSTμ, to breast cancer MCF7 cells had no apparent effect on resistance to DOX and CDDP (48). These results strongly suggested that GSTπ is important for metabolizing DOX and CDDP. Overexpression of GSTπ in cancer cells caused an acquisition of resistance to DOX and CDDP (35–38), but no change was observed after transfection (39). These experiments addressed the role of GSTπ in intracellular metabolism of anticancer drugs. However, there is no report on the role of nuclear GSTπ in anticancer drugs that target nuclear DNA. Furthermore, overexpression of GSTπ was studied in whole cells without the intracellular shift in location being addressed.

Ours is the first report on the importance of nuclear GSTπ in drug resistance. We observed the presence of nuclear GSTπ in various cancer cell lines. The amount of nuclear GSTπ on immunoblots did not depend on the amount in cytoplasm (Fig. 1). The results strongly suggest the existence of a specific transport system rather than simple diffusion for the nuclear transfer of GSTπ. The cancer cells in which no apparent GSTπ was found in the nucleus were sensitive to DOX and to apoptosis (Fig. 2). We speculated that nuclear GSTπ has an antiapoptotic role in regulating cell sensitivity to DOX.

GST activity was increased by treatment with anticancer drugs for 6 h (Table 1). It is strongly suggested that GST mRNA is induced by these anticancer drugs, leading to an increase in GST activity in the cytoplasm. Therefore, the possibility cannot be ruled out that such an increase in cytoplasmic GST causes its accumulation in the nucleus. However, in the preliminary study, production of ROS was observed in the cells treated with these anticancer drugs (data not shown). In HCT8 cells, only GSTπ was expressed, whereas other major subclasses of GST were not found. Next, we examined the effect of hydrogen peroxide on the nuclear transfer of GSTπ over a short period of incubation, during which stimulation of the expression of GSTπ mRNA cannot be detected. Treatment of cells with 200 μM hydrogen peroxide for 30 min increased the amount of nuclear GSTπ (Fig. 5*C, D, F*). These findings suggest that regulation of the nuclear transfer of GSTπ is independent of the amount of GSTπ in cytoplasm.

Next, we studied the mechanism of the nuclear transfer of GSTπ and the effect of nuclear GSTπ on drug resistance. We employed cancer cells in which nuclear GSTπ was induced by DOX, CPT-11, and CDDP, and did not examine the mechanism by which the transfer of GSTπ was prevented in some cancer cells. In the amino acid sequence of GSTπ, no transfer signal was found: a monopolar type of NLS composed of a group of basic amino acids found in SV40 T antigen, a bipolar type of NLS composed of two basic amino acids found in nucleoplasmin, or a shuttling sequence found in RNA binding protein known as the

DNA cross-linking by CDDP. The effect of GSTπ in the nucleus on DNA intercalation by DOX and DNA cross-linking by CDDP was studied in vitro by using a pUC-γ-GCS plasmid. DNA intercalation was analyzed by agarose gel electrophoresis. Various concentrations of DOX (0–20 μM) were incubated in a mixture in the presence or absence of GSTπ (0–3 U) and a GSH mixture (2 mM GSH, 2 U glutathione reductase, and 2 mM NADPH) for 30 min at 37°C and then were treated with 400 ng of the pUC-γ-GCS plasmid for 30 min. Various concentrations of CDDP (0–100 μM) were incubated in the presence or absence of GSTπ (0–3 U) and the GSH mixture for 1 h at 37°C and then were treated with 400 ng of the pUC-γ-GCS plasmid for 3, 6, or 12 h. Each 200 ng of the pUC-γ-GCS was applied to a 1% agarose gel for electrophoresis at 15 mA for 40 min. Final detection was with ethidium bromide (0.5 μg/ml).



M9 sequence in the heterogeneous nuclear ribonucleoprotein A1 (49). The phosphorylation of serine residues near the nuclear export signals in the amino acid sequence can reversibly act as an NLS, as reported for the nuclear accumulation of human cyclin B1 (50). The presence of such a mechanism cannot be ruled out in GSTπ, but electrophoretic patterns obtained by Western blot analysis revealed no apparent change in GSTπ in the nucleus compared with that in the cytoplasm (Fig. 3).

It has been reported that wheat germ agglutinin (WGA) inhibits the nuclear transfer of proteins when it enters cells (51–55). Because plasma membranes are not permeable to WGA, in previous experiments it was used by pretreatment of cells with digitonin to increase membrane permeability (51, 52), or by microinjection (53, 54). WGA was also used to prepare the nucleus (55). Yu et al. reported that ABL was efficiently internalized into the cytoplasm of cultured cells and localized around the nucleus and that it inhibited the nuclear transfer of proteins (44). This suggests that artificial modifications of cell membranes are required for the internalization of WGA, whereas ABL does not require any modifications. Thus, we used ABL to inhibit the nuclear transfer of GSTπ in cancer cells.

ABL inhibited the nuclear transfer of GSTπ, but it did not inhibit the nuclear transfer of p53, which possesses NLS (56) (Fig. 5*E*). These findings ruled out the possibility that the inhibitory effects of ABL on the nuclear transfer of proteins are nonspecific. Pretreatment of the cells with ABL decreased the steady-state level of the nuclear GSTπ. Furthermore, ABL inhibited the accumulation of nuclear GSTπ on exposure to DOX (Fig. 6). These findings suggest that GSTπ is transferred to the nucleus through a protein transfer system both in the steady state and after exposure to anticancer drugs. Because various mechanisms are proposed for the protein transfer to the nucleus, it is possible that if GSTπ possesses an unknown NLS, it is transferred to the nucleus through an unknown system or cotransferred with other proteins. The accumulation of GSTπ in response to DOX may be due to inhibition of the nuclear export system, similar to the inhibition under oxidative stress observed in fission yeast (57). In the present study, the precise mechanism was not clarified. Further study is needed.

Treatment of cancer cells with ABL also caused an increase in the accumulation of nuclear DOX (Fig. 7). Because of the method employed for the preparation of the nucleus, the DOX in the nucleus may reflect the amount bound to DNA. A cytotoxicity assay revealed that the nuclear GSTπ protects against the cytotoxicity of DOX (Fig. 6). Further study of DNA intercalation in vitro clearly demonstrated the protective effect of GSTπ on DNA intercalation by DOX (Fig. 8). pUC-γ-GCS employed as a plasmid DNA is not specific for intercalation by DOX. Similar results were observed using another plasmid constructed with cDNA coupled with pUC19 (data not shown). DOX and CDDP altered the electrophoretic pattern of pUC-γ-GCS, whereas GSTπ restored it, suggesting that GSTπ together with GSH functions to prevent DNA intercalation by DOX and DNA cross-linking by CDDP.

With regard to the effect of DOX on cancer cells, the involvement of ROS has been suggested (19). DOX attacks the nuclear components and ROS further damage the DNA. Because rat GSTπ possesses GSH peroxidase activity to scavenge peroxidized lipids and the nucleic acids (3–5), human GSTπ may scavenge DOX-induced peroxidation products in the nucleus.

In our experiments, CDDP induced apoptosis in HCT8 cells pretreated with ABL (data not shown). This result suggests an important role for nuclear GSTπ in protecting DNA from damage. Similarly, CPT-11

caused apoptosis in the cells treated with ABL (data not shown). The metabolic pathway of CPT-11 has not been elucidated, and the role of nuclear GSTπ in CPT-11-induced DNA damage is unclear at present.

Collectively, our findings show that GSTπ is transferred to the nucleus on exposure of the cell to anticancer drugs and prevents drug-induced DNA damage. The use of inhibitors targeting the nuclear transfer of GSTπ may prove an efficient therapy to reduce drug resistance in cancer cells. FJ

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